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SABATH methyltransferases from white spruce (*Picea glauca*): gene cloning, functional characterization and structural analysis

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Summary Known members of the plant SABATH family of methyltransferases have important biological functions by methylating hormones, signalling molecules and other metabolites. While all previously characterized *SABATH* genes were isolated from angiosperms, in this article, we report on the isolation and functional characterization of *SABATH* genes from white spruce (*Picea glauca* [Moench] Voss), a gymnosperm. Through EST database search, three genes that encode proteins significantly homologous to known SABATH proteins were identified from white spruce. They were named *PgSABATH1*, *PgSABATH2* and *PgSABATH3*, respectively. Full length cDNAs of these three genes were cloned and expressed in *Escherichia coli*. The *E. coli*-expressed recombinant proteins were tested for methyltransferase activity with a large number of compounds. While no activity was detected for PgSABATH2 and PgSABATH3, PgSABATH1 displayed the highest level of catalytic activity with indole-3-acetic acid (IAA). PgSABATH1 was, therefore, renamed PgIAMT1. Under steady-state conditions, PgIAMT1 exhibited apparent K_m values of 18.2 μM for IAA. Homology-based structural modelling of PgIAMT1 revealed that the active site of PgIAMT1 is highly similar to other characterized IAMTs from angiosperms. *PgIAMT1* showed expression in multiple tissues, with the highest level of expression detected in embryonic tissues. During somatic embryo maturation, a significant reduction in *PgIAMT1* transcript levels was observed when developing cotyledons become apparent which is indicative of mature embryos. The biological roles of white spruce *SABATH* genes, especially those of *PgIAMT1*, and the evolution of the *SABATH* family are discussed.

Keywords: gene expression, high-throughput enzyme assay, indole-3-acetic acid, somatic embryogenesis.

Introduction

Plants produce a large number of low molecular weight metabolites. Some of these metabolites, such as indole-3-acetic acid (IAA), gibberellic acid (GA), salicylic acid (SA) and jasmonic acid (JA), play critical roles in diverse biological processes ranging from plant growth and development to plant interactions with the environment (Taiz and Zeiger 2006). Chemical modifications of such metabolites may have a profound impact on their activities. For example, recent studies showed that IAA, GA, SA and JA undergo the same type of novel modification: methylation of their free carboxyl group. Methylation of these compounds may affect the concentrations of their free acids in plant tissues. In addition, the methylated products may have biological and ecological functions different from their precursors. The enzymes that catalyze the methylation of IAA, GA, SA and JA were discovered to be IAA methyltransferase (IAMT; Qin et al. 2005, Zhao et al. 2007, 2008), GA methyltransferase (GAMT; Varbanova et al. 2007), SA methyltransferase (SAMT; Ross et al. 1999, Chen et al. 2003) and JA methyltransferase (JMT; Seo et al. 2001), respectively.

Although IAMT, GAMT, SAMT and JMT methylate substrates of distinct chemical structures, they are homologous at the protein sequence level and belong to the same

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protein family called SABATH (D'Auria et al. 2003). In addition to the aforementioned SABATH proteins, other known SABATH proteins catalyzing carboxyl methylation include benzoic acid methyltransferase (BAMT; Murfitt et al. 2000), farnesoic acid methyltransferase (FAMT; Yang et al. 2006) and cinnamate/ρ-coumarate carboxyl methyltransferase (CCMT; Kapteyn et al. 2007). In addition to carboxyl methyltransferases, the SABATH family includes a number of nitrogen-directed methyltransferases involved in caffeine biosynthesis that are homologous to carboxyl SABATH methyltransferases (Ogawa et al. 2001). These include xanthosine methyltransferase, 7-methylxanthine methyltransferase (or therbromine synthase) and 3,7-methylxanthine methyltransferase (or caffeine synthase; Ashihara et al. 1996). A number of genes encoding these enzymes have been isolated from tea (Kato et al. 2000, Yoneyama et al. 2006) and coffee plants (Ogawa et al. 2001, Mizuno et al. 2003).

Before this study, all characterized *SABATH* genes were isolated from angiosperms. The presence of *SABATH*-like sequences in gymnosperms was noted in one of our previous studies (Zhao et al. 2007). None of the gymnosperm *SABATH* genes, however, has been functionally characterized. Functional studies of *SABATH* genes in gymnosperms may provide important insights into the evolution of the *SABATH* family as well as the biological processes that they control. Spruce is a genus widely distributed and used for forest tree plantations across the northern hemisphere. Extensive information has been accumulated on the genetic control of commercially important traits in spruce due to the efforts on genetic improvement. In recent years, white spruce (*Picea glauca* [Moench] Voss) has been used as a model for genomic study of spruce (Pavy et al. 2005). The extensive amount of genomic information combined with well-studied biology makes white spruce a useful model for studying the molecular biology and biochemistry of the SABATH family in gymnosperms.

Some characterized angiosperm *SABATH* genes play important biological roles. In *Arabidopsis*, *IAMT* was demonstrated to be involved in leaf development (Qin et al. 2005). *Arabidopsis* contains two *GAMT* genes, both of which have a role in seed development and germination (Varbanova et al. 2007). Both *Arabidopsis JMT* and *SAMT* are involved in plant defense responses (Seo et al. 2001, Chen et al. 2003). The substrates of aforementioned *SABATH* genes are known to play pivotal biological roles in both angiosperms and gymnosperms. Therefore, it will be particularly interesting to determine whether gymnosperms contain *SABATH* genes involved in the methylation of the same plant hormones and signalling molecules that are substrates of known angiosperm SABATH proteins.

Here, we report on the identification of *SABATH* genes from white spruce through analysis of EST databases and analysis of their relation with their angiosperm counterparts. We also report on the identification of substrates for white spruce SABATH proteins and the characterization of the biological functions of their encoding genes. One of the genes, *PgIAMT1*, was determined to encode IAMT. *PgIAMT1* showed dynamic changes in expression during in vitro somatic embryo maturation, indicating that this gene may play a role in embryogenesis, probably through modulating the homeostasis of IAA.

Materials and methods

Plant materials and chemicals

White spruce tissues were taken from 33-year-old trees from a progeny trial established near Québec City (Canada; according to Bedon et al. 2007), except for embryonic tissues. All tissues were frozen in liquid nitrogen immediately upon removal from the tree and stored at −80 °C until further use. We collected newly formed needles from the upper crown. Differentiating secondary xylem and phloem were collected from three 30–40-cm bolts taken from the lower third of the main stem. These vascular tissues were scraped with a scalpel immediately after peeling the bark. The tissues scraped from the exposed inner side of the bark and from the surface of the exposed wood were labelled as differentiating secondary phloem and xylem, respectively. Similarly, differentiating xylem was collected from large roots located in a 1 m radius from the base of the stem and the root tips were taken at the end of fine non-lignified roots. Embryonic tissue samples were taken from line PG653 on culture media or following the transfer to maturation media. The culture and maturation media as well as conditions for embryonic tissue culture were as described by Klimaszewska et al. (2004). For auxin treatments, the only variant was the replacement of dichlorophenoxyacetic acid (2,4-D) by IAA at different concentrations. For stress experiments, 4-month-old clonal trees (PG653) were mechanically wounded at the apex with forceps or treated with JA as previously described (Pervieux et al. 2004). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise.

Sequence retrieval and analysis

The protein sequence of *Clarkia breweri* SAMT (CbSAMT, Accession No. AF133053) was used initially as a query sequence to search against the white spruce EST database (ForestTreeDB; Pavy et al. 2007) using the tblastn algorithm (Altschul et al. 1990). The newly identified SABATH-like sequences were used reiteratively to search the same sequence database. The cutoff e value was set to be e^{-6}. Multiple protein sequence alignments were constructed using ClustalX software (Thompson et al. 1997) and displayed using GeneDoc (http://www.psc.edu/biomed/genedoc/). The phylogenetic tree was produced using ClustalX and viewed using the TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Cloning of coding sequences into Escherichia coli expression vector

Primers corresponding to the beginning and to the end of the coding sequences of the three white spruce *SABATH* genes were designed (Table 1) and used in polymerase chain reaction (PCR) to amplify the full-length cDNA of the three genes. The PCR products were cloned into pCRT7/CT-TOPO vector (Invitrogen, Carlsband, CA) and fully sequenced.

High-throughput biochemical assays

A high-throughput biochemical procedure modified from a previous report (Yang et al. 2006) was used in the initial screening of potential substrates for individual white spruce proteins. A total of 68 compounds (Table 2) containing a carboxyl group or nitrogen that theoretically could be methylated by SABATH proteins were tested. Each compound was dissolved in the appropriate solvent based on its chemical properties to make a stock solution (50 mM). These compounds were pooled into 14 groups, each containing two to six compounds. Fourteen assays were set up, each at a total volume of 50 µl containing 50 mM Tris–HCl pH 7.5 buffer, 5 µl protein, 0.5 µl [^{14}C]-SAM (specific activity 52.7 mCi mmol^{-1}, Perkin Elmer Instruments, Shelton, CT) and 1 µl substrates from a given pool. The reactions were incubated at 25 °C for 30 min and then extracted with 180 µl ethylacetate. The upper organic phase was counted using a liquid scintillation counter (Beckman Coulter, Fullerton, CA) as previously described (D'Auria et al. 2003).

Purification of recombinant PgIAMT1

The forward primer 5′-CACCATGGCTTTCAACTTCAA GACTGTTTGT-3′ and the reverse primer 5′-CTATTT TCTGGTAAGTGCAGCCACAA-3′ were used to subclone *PgIAMT1* cDNA into pET100/D-TOPO vector (Invitrogen, Carlsband, CA). To express PgIAMT1 protein in *E. coli*, the plasmid pET100/D-TOPO containing *PgIAMT1* full length cDNA was transformed into *E. coli* strain BL21 DE3 codon plus (Invitrogen, Carlsband, CA). Protein expression was induced by isopropyl β-D-1-thiogalactopyranoside for 18 h at room temperature and the cells were lysed by sonication. His-tagged PgIAMT1 protein was purified from *E. coli* cell lysate using Ni-NTA agarose following the manufacturer's instructions (Invitrogen, Carlsband, CA). The concentration of the protein was determined by the Bradford assay (Bradford 1976).

Biochemical characterization of PgIAMT1

Radiochemical IAMT assays were performed with 50 µl volume containing 50 mM Tris–HCl, pH 7.5, 1 mM IAA and 0.4 µl [^{14}C]-SAM. The assay was started by the addition of [^{14}C]-SAM and kept at 25 °C for 30 min. The radioactivity of the extracted assay products was measured using a liquid scintillation counter. The assays for PgIAMT1 with IAA, abscisic acid (ABA), GA, indole-3-butylic acid (IBA), 2,4-D and indole were the same as that for IAA except that the corresponding substrates were used in individual assays. Three independent assays were performed for each compound. Determination of detailed biochemical properties of PgIAMT1 including kinetic parameters, pH optimum and optimum temperatures as well as the identification of the product of IAMT assay using IAA as substrate were performed following the procedures previously described (Zhao et al. 2007).

Structural modelling

A homology model of the structure of PgIAMT1 was made based on the known structure of *Arabidopsis* IAMT (AtIAMT1; Zhao et al. 2008). First, a sequence alignment of AtIAMT1 and PgIAMT1 was performed with Blast using the Blosum62 matrix. Then, the homology model was calculated with the program Modeller (Sali and Blundell 1993) by generating a first model (3D alignment on template) and running 200 cycles of molecular dynamics annealing. The PgIAMT1–IAA complex was built by hand based on the position of the carboxyl group of IAA in the AtIAMT1 structure and rotating bonds to avoid steric clashes.

RNA extractions and gene expression analysis via real-time PCR

Frozen material was ground to fine powder using liquid nitrogen cooled 50 ml jars and 25 mm bead in a Mixer Mill MM300 engine (Retsch, Germany). The RNA extraction was performed as previously described for white spruce (Bedon et al. 2007). The RNA integrity was checked with a 2100 Bioanalyser (Agilent Technologies, Santa Clara).

Table 1. Primers used in this paper.

Analysis	Gene	Forward primer	Reverse primer
Full-length cDNA cloning	*PgIAMT1*	5′-ATGGCTTTCAACTTCAAGACTGTTTGT-3′	5′-CTATTTTCTGGTAAGTGCAGCCACAA-3′
	PgSABATH2	5′-ATGGACACAGTGAAATCAATGGAGAATG-3′	5′-CTATTTCCGAACAAGAAAAGCTGAAACC-3′
	PgSABATH3	5′-ATGGAGACGGTAAACACCCTCGAGAAT-3′	5′-TCATTTGCGAACCAGAAAGGCAGC-3′
Quantitative PCR	*PgIAMT1*	5′-TAAGCGAAAAGCTCTTCAACAGAATG-3′	5′-TTACAAGTATTGGATTGGATTGGACTG-3′
	PgSABATH2	5′-CGAGAATCTTGTGAGAGCTCAGTTG-3′	5′-TTCTACAATCCCACAGAGCGAAATG-3′
	PgSABATH3	5′-CACTTGGGGTGGGATGACTATCTG-3′	5′-GTTCAATTATACAACGCGAATGGAAC-3′
	PgDef.1	5′-CGATTTCCATGTAGCCAACAGAAAG-3′	5′-GAATCCCCAAACCAGAGAAGATCAC-3′

Table 2. Compounds used in high-throughput biochemical assays.

Group	Compounds	Solvent
1	Benzoic acid, caffeic acid, 3,5-dimethoxy-4-hydroxy-cinnamic acid, chlorogenic acid, phenylpyruvic acid	Ethanol
2	SA, ferulic acid, vanillic acid, gallic acid, 4-hydroxy-phenylpyruvic acid	Ethanol
3	3-Hydroxy-benzoic acid, anthranillic acid, *p*-coumaric acid, JA, shikimic acid	Ethanol
4	4-Hydroxy-benzoic acid, 4-amino benzoic acid, cinnamic acid, 3-hydroxy-4-methoxy-cinnamic acid, rosmarinic acid, 4-hydroxy-phenyllactic acid	Ethanol
5	ABA, GA, IAA, IBA, dichlorophenoxyacetic acid, indole	Ethanol
6	Zeatin, kinetin, 6-benzylaminopurine, γ-amino butyric acid, 3,4-dihydroxyphenylalanine, tryptophan	DMSO
7	Trigalacturonic acid, muranic acid	DMSO
8	Octanoic acid, decanoic acid, lauric acid, myristic acid, palmitic acid, stearic acid	Ethanol:chloroform (1:1)
9	Xanthosine, 7-methyl xanthine, theobromine, paraxanthine, β-alanine, camalexin	DMSO
10	Glutamic acid, valine, alanine, phenylalanine, aspartic acid, asparagine	H_2O
11	Nicotinic acid, nicotinamide, anabasine, nornicotine, tryptamine, tyramine	Ethanol
12	Farnesoic acid, geranic acid	Ethanol
13	Traumatic acid, 11-methyl-lauric acid, quinaldic acid, picolinic acid	Ethanol
14	Ascorbic acid, folic acid, pantothenic acid	H_2O

The cDNA was prepared from 500 ng of total RNA using the first strand cDNA synthesis system (Invitrogen, Carlsbad). The PCR mixtures containing QuantiTect SYBR Green PCR Kit (QIAGEN, Germantown) were as follows: 1× master mix, 300 nM of 5′ and 3′ primers and target DNA or cDNA in a final volume of 15 µl. Reactions were set up using an EpMotion 5075 pipetting robot (Eppendorf, Hamburg, Germany) and amplifications were carried out in a LightCycler 480 (Roche, Mannheim, Germany). Cycling for QuantiTect was performed as follows: an initial 15-min activation step at 95 °C, followed by 50 cycles of 94 °C for 10 s and 62 °C for 2 min and a single fluorescent read was taken after each cycle immediately following the annealing and elongation step at 62 °C. A melting curve was performed after each quantitative PCR (qPCR) experiment to ensure single product amplification of the appropriate melting temperature.

The methodology described here for the quantification of the number of transcripts is a slight modification of the procedure elaborated by Rutledge and Stewart (2008):

$$F_0 = \frac{F_{\max}}{1 + (E_{\max} + 1)^{C_{1/2}}}, \quad (1)$$

$$F_{\max} = \frac{E_{\max}}{-\Delta E}. \quad (2)$$

Insertion of Eq. (2) into (1), both described in Rutledge and Stewart, served to derive a new Eq. (3) used to quantify molecules.

$$F_0 = \frac{E_{\max}}{-\Delta E\left(1 + (E_{\max} + 1)^{C_{1/2}}\right)}. \quad (3)$$

In these equations, F_0 is the initial target quantity expressed in fluorescence units, $F_{\max}$ is the maximal fluorescence where the efficiency reaches 0, $E_{\max}$ is the maximal efficiency occurring at the beginning of cycling, $C_{1/2}$ is located at the inflexion point of the fluorescence curve where the fluorescence is half of $F_{\max}$ and the efficiency is half of $E_{\max}$ and ΔE represents the rate of loss in efficiency. For each amplification reaction, ΔE and $E_{\max}$ were determined using the linear regression of efficiency (LRE) method (Rutledge and Stewart 2008) and $C_{1/2}$ was calculated by taking the first derivative of the fluorescent reads. The F_0 were then transformed to molecules (N_0) with equations described in Rutledge and Stewart (2008). Fluorescence background was removed before LRE analysis and $C_{1/2}$ determination. Optical calibrations of the mastermixes in the LC480 were performed with lambda. An Excel spreadsheet designed to accommodate the 384 sample output from the LC480 was created to automatically convert fluorescent reads to molecules. Sequences of the primer are shown in Table 1.

Results

Identification and sequence analysis of SABATH genes from white spruce

The EST databases constitute a valuable resource to survey the species in which a gene is present and expressed. When the white spruce EST database (ForestTreeDB; Pavy et al. 2007) was searched using CbSAMT as a tblastn query, 11 unigene sequences encoding proteins significantly homologous to CbSAMT were identified. Comparison of gene structures revealed that three of the 11 unigenes are full-length. They were named *PgSABATH1*, *PgSABATH2* and *PgSABATH3*, encoding proteins of 403 aa, 389 aa and 389 aa, respectively. PgSABATH1 has a longer N-terminal compared to PgSABATH2 and PgSABTAH3 (Figure 1).

To understand the evolutionary relationship between the white spruce SABATH proteins and known SABATH

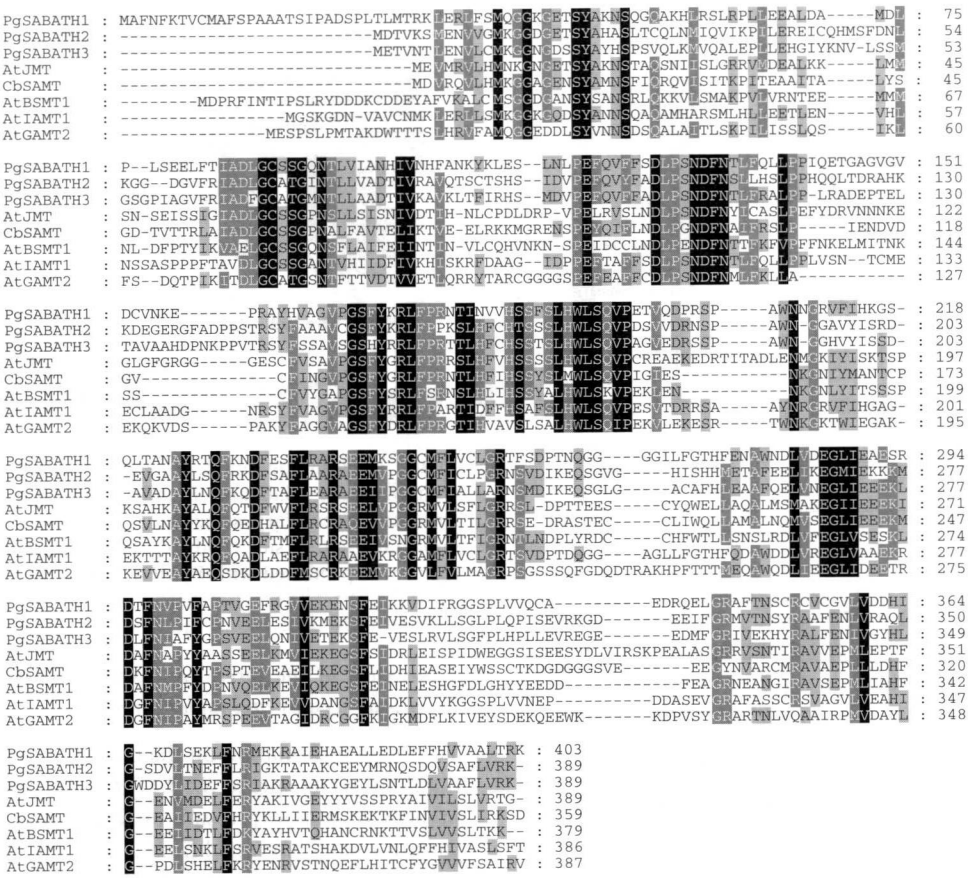


Figure 1. Sequence alignment of white spruce SABATH proteins (PgSABATH1, PgSABATH2 and PgSABATH3) and selected known SABATH proteins including AtJMT, CbSAMT, AtBSMT1 (*Arabidopsis* benzoic acid/salicylic acid MT), AtIAMT and AtGAMT2 (*Arabidopsis* GAMT2).

proteins, a phylogenetic tree was constructed consisting of PgSABATH1, PgSABATH2, PgSABATH3, rice IAMT (OsIAMT1), poplar IMAT (PtIAMT1), snapdragon BAMT (AmBAMT), all *Arabidopsis* SABATHs, coffee caffeine synthase (CCS1) and one putative SABATH protein from pine. PgSABATH1 belongs to the clade that contains AtIAMT1, PtIAMT1 and OsIAMT1. In contrast, PgSABATH2 and PgSABATH3 cluster in a clade that also contains the pine SABATH protein (Figure 2).

High-throughput biochemical analysis of white spruce SABATH proteins

In the high-throughput biochemical screening, PgSABATH1 showed activity with compounds in group 5 (Table 2). The PgSABATH2 and PgSABATH3 did not show activity with any of the substrate groups tested. Group 5 consists of six compounds, including ABA, GA, IAA, IBA, 2,4-D and indole. Additional methyltransferase enzymatic assays with individual compounds from this group using purified PgSABATH1 indicated that PgSABATH1 had the highest level of catalytic activity with IAA. The enzyme also displayed activity with IBA and 2,4-D, which was 20% and 4.3% of the activity observed with IAA, respectively. PgSABATH1 had no activity with ABA, GA and indole. The PgSABATH1 was, therefore, renamed PgIAMT1.

Biochemical properties of PgIAMT1

Purified PgIAMT1 recombinant protein was characterized in greater detail. To determine pH optimum, PgIAMT1 was assayed with IAA at buffers with different pH ranging from pH 6.5 to 10. The optimum pH was determined to be pH 7.5. At pH 6.5, the activity was about 15% of the optimum. At pH 8, the activity was reduced to about 70% of the optimum activity. The activity was reduced to 10% when the pH was changed to 9.5 and a complete loss of activity occurred at pH 10. Similarly, standard IAMT assays were carried out at different temperatures ranging from 4 to 50 °C, which revealed that an optimum temperature for PgIAMT1 is 25 °C.

Kinetic parameters for PgIAMT1 were also determined. Under steady-state conditions, PgIAMT1 exhibited K_m values of 18.2 and 22.7 μM for IAA and SAM, respectively, and a k_{cat} for IAA is 0.08 s^{-1}. The calculated catalytic efficiency of PgIAMT1 is 4414 s^{-1} M^{-1}.

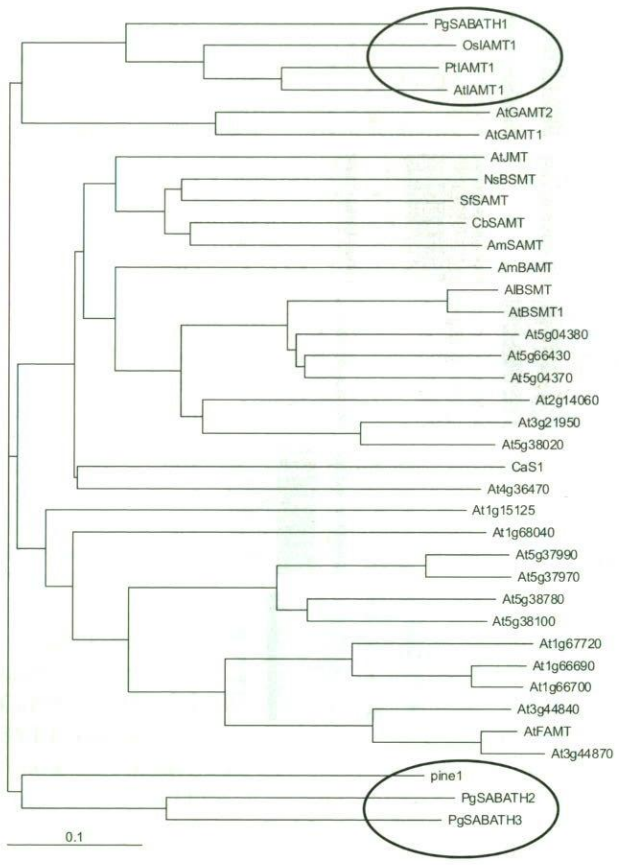


Figure 2. A neighbor-joining tree constructed with white spruce SABATH proteins and known SABATH proteins. CbSAMT (AF133053); AmSAMT, *Antirrhinum majus* L. (snapdragon) SAMT (AF515284); SfSAMT, *Stephanotis floribunda* SAMT (AJ308570); AmBAMT, *A. majus* BAMT (AF198492); NsBSMT, *Nicotiana suaveolens* Lehm. BSMT (AJ628349); AtBSMT1 (BT022049); AlBSMT, *Arabidopsis lyrata* L. BSMT (AY224596); AtJMT (AY008434); AtFAMT, *Arabidopsis thaliana* FAMT (AY150400); OsIAMT1, *Oryza sativa* L. IAMT1 (EU375746); PtIAMT1, *Populus trichocarpa* (Torr. & Gray) IAMT1 (fgenesh4_pg.C_LG_I002808); AtIAMT1, *A. thaliana* IAMT (AK175586); AtGAMT1, *A. thaliana* GAMT1 (At4g26420); AtGAMT2, *A. thaliana* GAMT2 (At5g56300); Cas1, *Coffea arabica* L. caffeine synthase 1 (AB086414); pine 1 represents a SABATH-like protein identified from pine. All others are SABATH proteins from *Arabidopsis*. The two clades containing white spruce SABATHs are circled. Branches were drawn to scale with the bar indicating 0.1 substitutions per site.

Structurally guided modulation of PgIAMT1

A homology model of the substrate pocket of PgIAMT1 (Figure 3) was built with Modeller (Sali and Blundell 1993) based on the experimentally determined AtIAMT1 structure. The model shows a high degree of similarity of the overall structure among PgIAMT1 and AtIAMT1, which is a predictable consequence of the protein sequence similarity. Noticeably, the PgIAMT1 model exhibits the hydrophobic residues that form the substrate binding site previously observed in AtIAMT1 (Phe158, Leu226, Leu242, Phe243, Val326 and Phe364 in AtIAMT1 sequence; Figure 3).

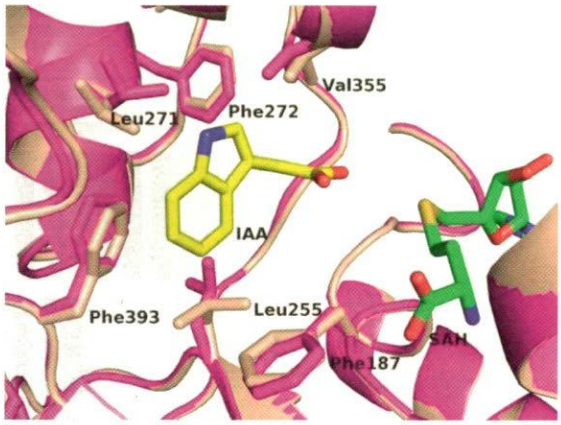


Figure 3. Homology model of PgIAMT1 active site, calculated with Modeller (Sali and Blundell 1993) based on AtIAMT1 structure. Secondary structure is represented as ribbons and important active site residues are represented as bonds. This figure was produced with PyMOL (http://www.pymol.org).

Expression of the three white spruce SABATH genes in various tissues and under stresses

To obtain information on the biological processes in which the three white spruce *SABATH* genes are involved, comprehensive gene expression analyses were performed with various tissues harvested from a mature tree and on embryonic tissue masses using qPCR. Both *PgSABATH2* and *PgSABATH3* showed the highest level of expression in root tips, while *PgIAMT1* showed the highest level of expression in embryonic tissues. Overall, the highest expression level observed was for *PgIAMT1*, reaching 150,000 transcript molecules per nanogram of total RNA in embryonic tissues, which was roughly six times its expression in xylem tissues (Figure 4). The abundance of the different *PgSABATHs* transcripts also varied significantly. For example, the *PgIAMT1* transcript level was more than 50-fold the level of the other two *PgSABATHs* in embryonic masses, and, by contrast, the expression level *PgSABATH2* was 10 times that of *PgIAMT1* and *PgSABATH3* in root tips. The highest transcript level of *PgSABATH3*, 8109 in root tips, is equivalent to about 10% and 5% of the highest expression of *PgSABATH2* (root tips) and *PgIAMT1* (embryonic tissues), respectively.

To evaluate potential changes in the expression of the three white spruce *SABATH* genes in response to stresses, the transcripts levels of *PgIAMT1*, *PgSABATH2* and *PgSABATH3* were determined using qPCR in seedlings treated with JA or mechanically wounded. Transcript accumulation was monitored 24 h after treatment in the terminal leader and in the lignified portion of the stem. The previously characterized defense gene *PgDef.1* (Pervieux et al. 2004) was used as a positive control for both JA and wounding treatments. The *PgIAMT1* transcripts were down-regulated in both conditions (Figure 5A). In contrast, there was no significant effect on *PgSABATH2* transcripts

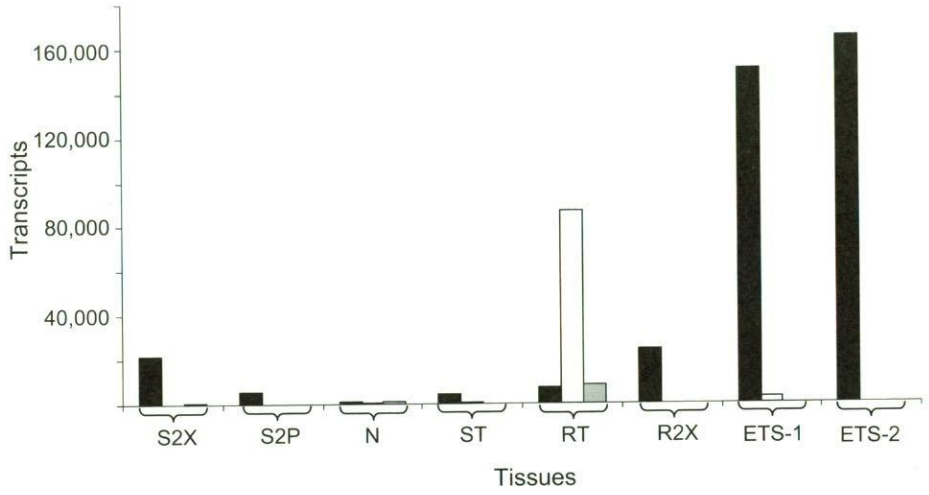


Figure 4. Tissue-specific expression of three white spruce *SABATH* genes. The expression of *PgIAMT1*(black), *PgSABATH2* (white) and *PgSABATH3* (grey) were measured in stem secondary xylem (S2X); stem secondary phloem (S2P); needles (N); shoot tip (ST); root tips (RT); root secondary xylem (R2X) and two independent samplings of embryonic tissue (ETS-1 and ETS-2). Expression is given as transcripts per nanogram of total RNA.

(Figure 5B), while *PgSABATH3* transcripts were significantly up-regulated in the terminal leader 24 h after wounding (Figure 5C).

Expression of PgIAMT1 during somatic embryo development

PgIAMT1 showed the highest level of expression in immature somatic embryonic tissues (Figure 4). To understand how *PgIAMT1* may be related to somatic embryo development, its expression was analyzed in the course of somatic embryo maturation. Immature embryonic culture was transferred from maintenance to maturation media to initiate the maturation process. These two media differ by the presence of 2,4-D and benzylaminopurine in maintenance medium, whereas the maturation medium contains ABA and increased sucrose content. At 14 days after transfer, the embryo proper increased in size (Figure 6A). At 21 days in maturation medium, root and shoot pole become well-developed (Figure 6B). At 28 days in culture, a ring of cotyledons is formed at the shoot apical region (Figure 6C and D), which is indicative of mature embryo formation.

To determine how *PgIAMT1* expression is associated with somatic embryo formation, RNA was extracted from somatic embryonic tissues at different developmental stages and the transcripts were analyzed by reverse transcriptase qPCR. The expression of *PgIAMT1* remained at a high level for the first 3 weeks post-transfer. A fivefold decrease in the expression of *PgIAMT1* was observed at 28 days post-transfer and its expression remained low for the remainder of the experiment (Figure 6E).

Expression of PgIAMT1 in IAA-treated embryonic tissues

In addition to being the substrate of IAMT, IAA is a plant hormone that regulates the expression of a large set of genes. To understand whether the level of IAA can regulate the expression of *PgIAMT1*, the expression of *PgIAMT1* in IAA-treated embryonic tissues was analyzed using reverse transcriptase qPCR. Embryonic tissue was transferred from culture media containing 2,4-D to culture media where 2,4-D was substituted with IAA at different concentrations and the expression of *PgIAMT1* was monitored 24 and 48 h after transfer (Figure 7). There was no significant change in the expression of *PgIAMT1* following the switch from 2,4-D to IAA even if the concentration of IAA was five times the original auxin concentration.

Discussion

We have cloned and characterized three white spruce genes that share significant sequence similarity with angiosperm members of the *SABATH* family. This is the first report, to our knowledge, on the isolation and functional characterization of *SABATH* genes from a gymnosperm. To identify potential substrates of the three white spruce SABATH proteins, we performed high-throughput biochemical assays. PgSABATH1 (PgIAMT1) was identified to encode IAMT. In contrast, PgSABATH2 and PgSABATH3 did not show activity with any of the substrates tested, suggesting that these two proteins methylate plant metabolites yet to be identified. In addition to the three *SABATH* genes that are analyzed in this paper, the white spruce genome contains at least eight other *SABATH* genes that showed expression under certain conditions. Biochemical functions of the proteins encoded by these genes cannot be assigned merely based on their sequence similarities to known SABATH proteins. Biochemical studies are needed to identify the substrates and the products of these white spruce SABATH proteins.

Indole-3-acetic acid, the substrate of PgIAMT1, is the most active endogenous auxin in plants. It is involved in diverse biological processes including cell elongation, cell division, gravitropism, apical dominance and lateral root formation (Leyser 2002, Teale et al. 2006). As a newly

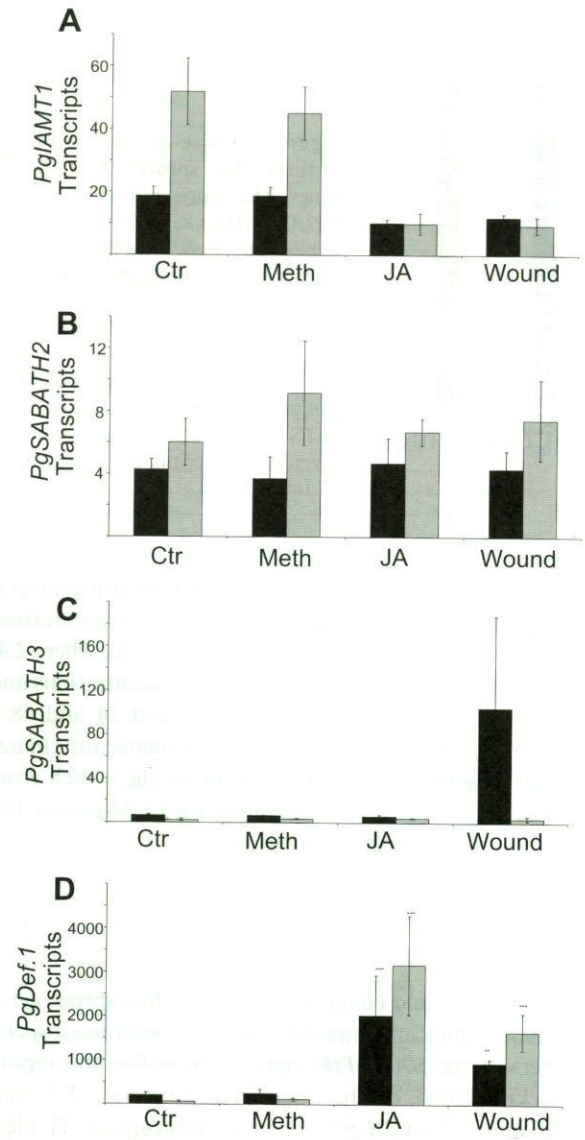
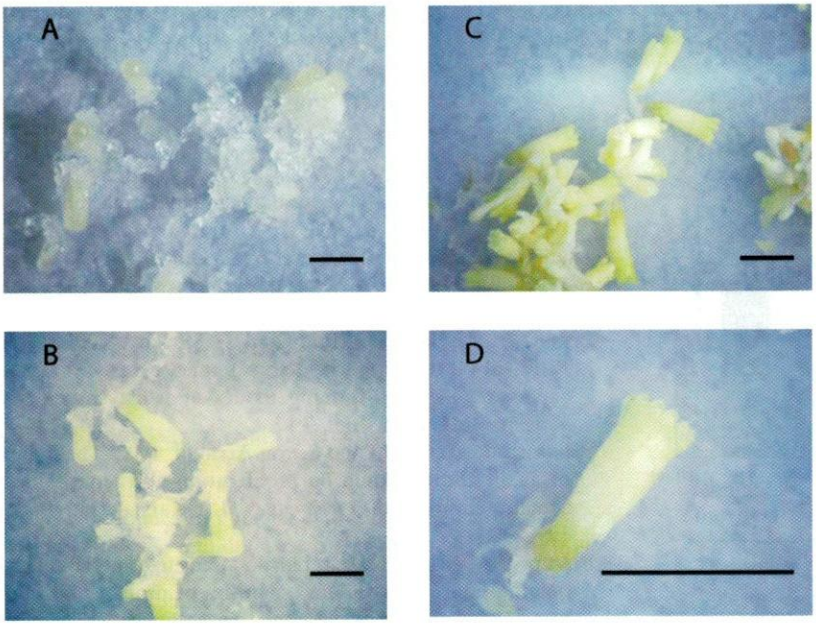


Figure 5. Expression of three white spruce *SABATH* genes under stress conditions. (A) *PgIAMT1*, (B) *PgSABATH2*, (C) *PgSABATH3* and (D) *PgDef.1* control gene. Four-month-old clonal material (PG653) was mechanically wounded (Wound) at the apex with forceps or treated with JA. Untreated plants (Ctr) and methanol-treated plants (Meth) serve as control for the wounding and JA experiments, respectively. Gene expression was determined in terminal leader (black) and lignified stem (grey) 24 h after treatments using qPCR. Expression is given as transcripts per nanogram of total RNA.

identified enzyme in plants, IAMT can modulate the homeostasis of IAA by converting IAA to its methyl ester. Before this study, *IAMT* genes have been isolated from three angiosperms, including herbaceous plants and trees: *Arabidopsis*, rice and poplar (Zhao et al. 2008). While the function of *IAMT* has not been fully characterized in rice and poplar, the *Arabidopsis IAMT* was shown to have a role in leaf development (Qin et al. 2005). The presence of *PgIAMT1* transcripts in a variety of tissues suggests that this gene is involved in multiple biological processes in white spruce, two of which are particularly noteworthy: embryogenesis and xylem formation.

The *PgIAMT1* transcript levels were highest in embryonic tissues (Figure 4) and changed during the course of somatic embryogenesis (Figure 6E). It took 28 days following the dramatic hormonal switch from growth promoting media containing auxin and cytokinin to maturation media containing ABA to observe a reduction in the expression of *PgIAMT1* (Figure 6). This is contrasted by the up, down and up again global gene expression pattern observed throughout the maturation process in Norway spruce (Van Zyl et al. 2003). Additionally, replacing 2,4-D by IAA or changing the auxin/cytokinin ratio did not alter the expression of *PgIAMT1* (Figure 7). Together, these results indicate that *PgIAMT1* expression is under developmental control rather than under direct hormone influence. Alternatively, it is possible that rapid transient changes in expression were missed because of the experimental design. Nonetheless, it has long been recognized that IAA plays an important role in embryogenesis (Mayer et al. 1991, Kong et al. 1997). For example, auxins and cytokinins are essential for proliferation and for maintaining the differentiation ability of embryonic cultures (Bellarosa et al. 1992). Consequently, to stimulate maturation of somatic embryos, it is necessary to transfer the embryonic cultures to medium lacking auxins (Von Arnold et al. 2002). In the somatic embryo of larch, the concentrations of endogenous free IAA remained at a low level in the early stage of somatic embryo formation, and increased dramatically at the late stage of somatic embryo maturation (Von Aderkas et al. 2001). The modulation of IAA contents may have a profound impact on the developmental program of embryos as it regulates the expression of a large set of genes (Stasolla et al. 2003). The levels of IAA can be regulated at different levels, including biosynthesis, conjugation/deconjugation and degradation. Methylation is a newly identified mechanism of IAA modulation. Recent studies suggest that methyl IAA is an inactive form of IAA and it can be converted back to IAA by the action of an esterase (Li et al. 2008, Yang et al. 2008). The high-level expression of *PgIAMT1* at the early stage of somatic embryogenesis and its low level of expression during maturation (Figure 6E) support that this gene may have an important role in embryogenesis by regulating IAA homeostasis. Analyses using large-scale expression profiling revealed dynamic patterns of gene expression changes during spruce somatic embryogenesis (Stasolla et al. 2003, Van Zyl et al. 2003). It will be particularly interesting to determine whether *PgIAMT1* has a role in regulating the expression of some of the embryogenesis-related genes.

PgIAMT1 also showed high levels of expression in secondary xylem and root xylem (Figure 4). The IAA has been demonstrated to be important for wood formation (Moyle et al. 2002). In poplar, a recent genomic study showed that auxin-responsive genes in wood-forming tissues respond



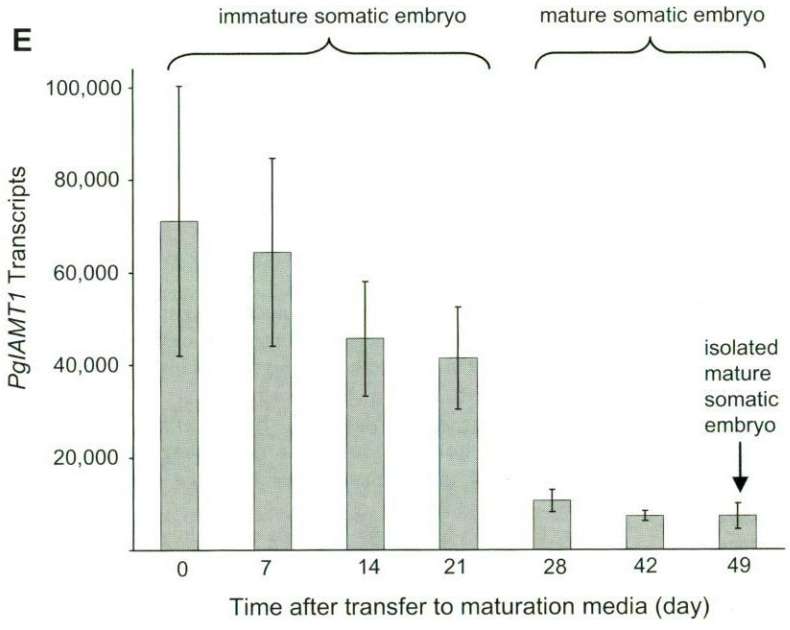


Figure 6. Maturation of white spruce somatic embryos and *PgIAMT1* expression. White spruce embryonic cell cultures were spread on filter paper and deposited onto the maturation media. Photographs were taken at (A) 14, (B) 21 and (C and D) 28 days after the transfer to maturation media. A clear reduction of the number of transparent suspensor cells is observed during the maturation process. The maturation process is completed at 28 days when cotyledons are clearly defined (D). The bar represents 2.5 mm. (E) Expression of *PgIAMT1* during somatic embryo maturation. White spruce embryonic cell cultures were spread on filter paper and deposited onto the maturation media. Tissues were collected weekly. Expression of *PgIAMT1* in collected tissues were determined using qPCR and are given as transcripts per nanogram of total RNA.

dynamically to changes in cellular IAA levels. These auxin-responsive genes modulate the expression of a few key regulators and in turn may control the global gene expression patterns that are essential for normal secondary xylem development (Nilsson et al. 2008). The high *PgIAMT1* expression level in secondary xylem suggests that this gene may be involved in regulating IAA homeostasis in xylem-forming tissues.

Although the biochemical activity of *PgSABATH2* and *PgSABATH3* are unknown, gene expression analysis suggests that these two genes have roles specific to root tips under normal growing conditions (Figure 4). Moreover, *PgSABATH3* appears to have a specific role in plant response to physical wounding (Figure 5). In addition to the three genes described in this paper, the white spruce genome contains other *SABATH* homologues. It will be interesting to isolate the full-length cDNAs of those genes and characterize the proteins they encode. Some of them may have similar biochemical functions as their counterparts in angiosperms. For example, methyl salicylate, which is produced by many angiosperm species for diverse purposes, is also produced by Norway spruce (Martin et al. 2003), a gymnosperm closely related to white spruce. The enzyme that catalyzes the formation of methyl salicylate

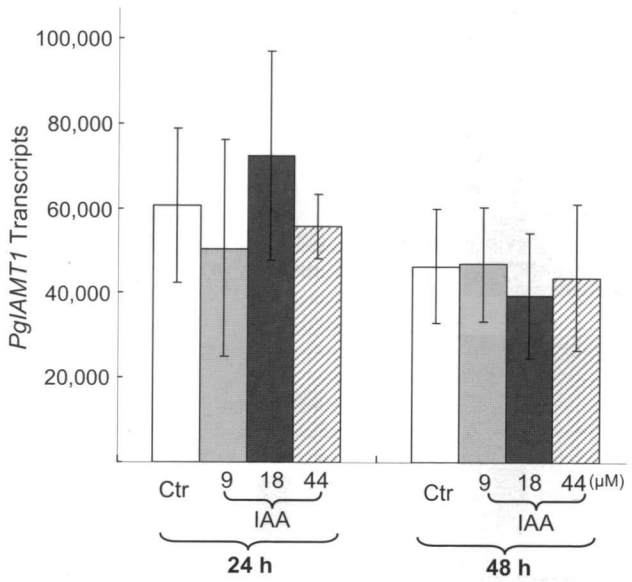


Figure 7. Expression of *PgIAMT1* in IAA treated somatic embryos. White spruce embryonic cell cultures were spread on filter paper and deposited onto culture media containing either three different concentrations of IAA (9, 18 or 44 μM) or 9 μM 2,4-D as control (Ctr). The *PgIAMT1* transcripts levels were monitored at 24 and 48 h after transfer using real-time PCR. Error bars represent standard deviation of the mean.

in Norway spruce is likely a member of the SABATH family.

Continued isolation and characterization of *SABATH* genes from gymnosperms will also help us understand substrate specificity evolution of the SABATH family, which methylate molecules of diverse structures. One of our recent studies on the SABATH family suggests that *IAMT* is an evolutionarily ancient member of the SABATH family in flowering plants (Zhao et al. 2008). The white spruce IAMT is highly homologous to angiosperm IAMTs at the protein sequence level and they exhibit comparable biochemical properties (Zhao et al. 2008). The active site of PgIAMT1 is also highly similar to that of AtIAMT1 (Figure 3). The presence of an IAMT in gymnosperms that is phylogenetically highly related to IAMTs from *Arabidopsis*, rice and poplar suggests that IAMT is an evolutionarily ancient member of seed plants. It will be interesting to determine whether *IAMT* genes are also present in non-seed plants, including vascular non-seed and non-vascular plants, which will provide further insights into the evolution and biological functions of *IAMT* and the SABATH family.

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